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01/14/02 U.S. PTO 60/348695

TITLE OF THE INVENTION (500 characters max)

INTRANASAL DELIVERY OF HIV ANTIGENS

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ENCLOSED APPLICATION PARTS (check all that apply)

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Respectfully submitted,

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Date 01/14/2002

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Docket Number	18892.001

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U.S. Provisional Patent Application Entitled

INTRANASAL DELIVERY OF HIV ANTIGENS

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HIV VACCINE AND METHOD OF USE

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is directed to pharmaceutical compositions comprising an HIV antigen and a mucosal adjuvant and methods for raising an immune response in a subject by administering these compositions. Preferably, the pharmaceutical compositions of the invention can be used to treat or prevent HIV infection.

HIV antigens suitable for use in this invention include envelope proteins such as gp120 and gp160 proteins, and antigenic fragments and derivatives thereof, such as oligomeric gp140 (Ogp140). Preferably, the antigens of the invention are optimized for immunogenicity.

The pharmaceutical compositions of this invention are suitable for mucosal delivery, preferably intranasal, intra-vaginal and intra-rectal delivery. Mucosal adjuvants suitable for use in this invention include detoxified mutants of *E. coli* heat labile toxin (LT), such as LTR72 and LTK63.

2. State of the Art

Acquired immune deficiency syndrome (AIDS) is recognized as one of the greatest health threats facing modern medicine and worldwide sexual transmission of HIV is the leading cause of AIDS. There are, as yet, no cures or vaccines for AIDS. Therefore, construction of a vaccine or drug that can specifically protect against sexual transmission at the site of entry is highly desirable.

In 1983-1984, three groups independently identified the suspected etiological agent of AIDS. *See, e.g.*, Barre-Sinoussi et al. (1983) Science 220:868-871; Montagnier et al., in Human T-Cell Leukemia Viruses (Gallo, Essex & Gross, eds., 1984); Vilmer et al. (1984) The Lancet 1:753; Popovic et al. (1984) Science 224:497-500; Levy et al. (1984) Science 225:840-842. These isolates were variously called lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), or AIDS-associated retrovirus

(ARV). All of these isolates are strains of the same virus, and were later collectively named Human Immunodeficiency Virus (HIV). With the isolation of a related AIDS-causing virus, the strains originally called HIV are now termed HIV-1 and the related virus is called HIV-2 See, e.g., Guyader et al. (1987) Nature 326:662-669; Brun-Vezinet et al. (1986) Science 233:343-346; Clavel et al. (1986) Nature 324:691-695. Consequently, there is a need in the art for compositions and methods suitable for treating and/or preventing HIV infection worldwide.

Although there is some discrepancy as to the effectiveness of cell-mediated or antibody-mediated responses in protection against disease, there is general consensus that generation of both cell-mediated and antibody-mediated responses is highly desirable. Antibody mediated responses would inhibit binding of the virus to its targets in vaginal or rectal tissues, *i.e.*, at the site of transmission, whereas cell-mediated responses would play a role in the eradication of infected cells.

Thus, as most HIV infections are transmitted through the female genital tract followed by systemic spread of the virus, induction of local as well as systemic immunity is greatly sought.

BRIEF SUMMARY OF THE INVENTION

This invention is directed to pharmaceutical compositions comprising an HIV antigen and a mucosal adjuvant and methods for raising an immune response in a subject by administering these compositions. Preferably, the pharmaceutical compositions of the invention can be used to treat or prevent HIV infection.

HIV antigens suitable for use in this invention include envelope proteins such as gp120 and gp160 protein, and antigenic fragments and derivatives thereof, such as oligomeric gp140 (Ogp140). Preferably, the antigens of the invention are optimized for immunogenicity.

The pharmaceutical compositions of this invention are suitable for mucosal delivery, preferably intranasal, intra-vaginal and intra-rectal delivery.

Mucosal adjuvants suitable for use in this invention include detoxified mutants of *E. coli* heat labile toxin (LT), such as LTR72 and LTK63.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to pharmaceutical compositions comprising an HIV antigen and a mucosal adjuvant and methods for raising an immune response in a subject by administering these compositions. The pharmaceutical compositions of this invention are suitable for mucosal delivery, preferably intranasal, intra-vaginal and intra-rectal delivery. Mucosal adjuvants suitable for use in this invention include detoxified mutants of E. coli heat labile toxin (LT), such as LTR72 and LTK63. In addition, the compositions of this invention can be used in combinations of mucosal prime/systemic boost or systemic prime/mucosal boost.

1. Pharmaceutical Compositions

The antigens used in this invention comprise antigens derived from HIV. Such antigens include, for instance, the structural proteins of HIV, such as Env, Gag and Pol. Preferably, the antigens of this invention comprise an HIV Env protein, such as gp140. Still more preferably, the antigens of this invention are optimized for immunogenicity and oligomerized, such as Ogp140.

The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins divided into three major classes: (1) the major structural proteins, Gag, Pol, and Env; (2) the regulatory proteins, Tat and Rev and (3) the accessory proteins, Vpu, Vpr, Vif, and Nef. Many variants are known in the art, including HIV_{SF2}, HIV_{IIIb}, HIV_{SF2}, HIV-1_{SF162}, HIV-1_{SF170}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}, HIV-1_{CM235}, HIV-1_{US4}, other HIV-1 strains from diverse subtypes (e.g., subtypes, A through G, and O), HIV-2 strains and diverse subtypes (e.g., HIV-2_{UC1} and HIV-2_{UC2}), and simian immunodeficiency virus (SIV). (See, e.g., Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991); *Virology*, 3rd Edition (Fields,

BN, DM Knipe, PM Howley, Editors, 1996, Lippincott-Raven, Philadelphia, PA; for a description of these and other related viruses).

In addition, due to the large immunological variability that is found in different geographic regions for the open reading frame of HIV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, at least eight different subtypes of HIV have been identified and, of these, subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa. Subtype C is also prevalent in India and it has been recently identified in southern Brazil. Subtype E was initially identified in Thailand, and is also present in the Central African Republic. Subtype F was initially described in Brazil and in Romania. The most recent subtypes described are G, found in Russia and Gabon, and subtype H, found in Zaire and in Cameroon. Group O viruses have been identified in Cameroon and also in Gabon. Thus, as will be evident to one of ordinary skill in the art, it is generally preferred to select an HIV antigen that is appropriate to the particular HIV subtype that is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, by sequencing the HIV genome (or fragments thereof) isolated from individuals within that region. Importantly, we have found that antibodies induced by immunizations with Ogp140 can neutralize various strains of HIV and therefore can be used as a prophylactic vaccine in several regions of the world.

As described above, also presented by HIV are various *Gag* and *Env* antigens. HIV-1 *Gag* proteins are involved in many stages of the life cycle of the virus including, assembly, virion maturation after particle release, and early post-entry steps in virus replication. The roles of HIV-1 *Gag* proteins are numerous and complex (Freed, E.O. (1998) *Virology* 251:1-15). For its part, the envelope protein of HIV-1 is a glycoprotein of about 160 kD (gp160). During virus infection of the host cell, gp160 is cleaved by host cell proteases to form gp120

and the integral membrane protein, gp41. The gp41 portion is anchored in (and spans) the membrane bilayer of virion, while the gp120 segment protrudes into the surrounding environment. As there is no covalent attachment between gp120 and gp41, free gp120 is released from the surface of virions and infected cells.

The sequences encoding the open reading frame of the ectodomain of the Env protein (gp140) from the HIV-1_{US4} strain were codon-optimized as described elsewhere [Haas, 1996 #562; zur Megede, 2000 #1451], and constructed synthetically as a 2.1 kb EcoR1-Xba1 DNA fragment (Midland Reagent Company, Midland, TX). This gene cassette contained the protein-encoding region of the Env protein fused in frame to the human tissue plasminogen activator (tPA) signal sequence as previously described [Chapman, 1991 #1550]. In order to stabilize the oligomeric structure of the encoded gp140 protein, the DNA sequence was mutated to introduce an arginine to serine change in the primary protease cleavage site (REKR) in the Env polypeptide [Earl, 1990 #2906](Fig.1A). The resulting Env expression cassette (gp140) was cloned into the EcoR1-Xba1 sites of the pCMV3 expression vector for the derivation of stable CHO cell lines. This vector contains the CMV enhancer/promoter elements, an ampicillin resistance gene, and sequences encoding a fusion protein composed of dihydrofolate reductase (DHFR) and an attenuated neomycin resistance protein.

At least one immunogenic portion of an HIV antigen may be used for mucosal immunization. As utilized herein, "immunogenic portion" refers to a portion of the respective antigen that is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). The immunogenic portion(s) used for immunization may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, or

ELISPOT, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al. (1991) *J. Exp. Med. 173*:1007-1015; Vitiello et al. (1992) *Abstract of Molecular Biology of Hepatitis B Virus Symposia*).

Additional immunogenic portions of the HIV antigens described herein may be obtained by truncating the coding sequence at various locations including, for example, to include one or more epitopes from the various domains of the HIV genome. As noted above, such domains include structural domains such as *Gag, Gag-polymerase, Gag-protease, reverse transcriptase (RT), integrase (IN)* and *Env*. The structural domains are often further subdivided into polypeptides, for example, p55, p24, p6 (*Gag*); p160, p10, p15, p31, p65 (*pol, prot, RT and IN*); and gp160, gp120 and gp41 (*Env*) or Ogp140 as constructed by Chiron Corporation. Additional epitopes of HIV and other sexually transmitted diseases are known or can be readily determined using methods known in the art. Also included in the invention are molecular variants of such polypeptides, for example as described in PCT/US99/31245; PCT/US99/31273 and PCT/US99/31272.

Preferably, the antigens of this invention are optimized for immunogenicity, such as Ogp140.

As used herein, the phrase "optimized" refers to an increase in the immunogenicity of the proteins, so that they can induce higher quantity and quality of antibodies. Moreover, polynucleotide sequences that can encode Ogp140 can be optimized by codon substitution of wild type sequences. Haas, et al., (Current Biology 6(3):315-324, 1996) suggested that selective codon usage by

HIV-1 appeared to account for a substantial fraction of the inefficiency of viral protein synthesis. Andre, et al., (J. Virol. 72(2):1497-1503, 1998) described an increased immune response elicited by DNA vaccination employing a synthetic gp120 sequence with optimized codon usage. Schneider, et al. (J. Virol. 71(7):4892-4903, 1997) discuss inactivation of inhibitory (or instability) elements (INS) located within the coding sequences of the Gag and Gag-protease coding sequences.

The sequences encoding codon-optimized gp140 were cloned into an expression vector for the evaluation of Env expression in transient transfection experiments and for protein purification. To facilitate the efficient secretion of recombinant Ogp140 protein, the native HIV signal sequence was replaced by the human tissue-type plasminogen activator (t-PA) signal sequence. The effect of codon optimization on gp140 expression was determined by transient transfection of 293 cells with codon-optimized and native (non-codon optimized) gp140 constructs and, comparison of expression levels by a capture ELISA and immunoblotting. It was shown previously that sequence modification of HIV gag dramatically improved the level of expression [zur Megede, 2000 #1451], similarly, codon optimization also improved the expression of gp140 4 to 10 fold compared to the native construct [Haas, 1996 #562]. Using such sequence-modified constructs we developed stable CHO cell lines secreting 5-15 µg/ml of o-gp140 and gp120. The antigenicity of oligomeric gp140 with and without a point mutation (R509 to S509) in the gp120/g41 primary protease cleavage site was also evaluated by transiently transfecting the 293 cells. Expression and structural characterization data indicated that the native form of the HIV-1 ectodomain-encoding region did not form gp140 oligomers efficiently (only about 50% of the expressed protein was found to be in oligomeric conformation). In contrast, the single R to S mutation in the protease cleavage site resulted in the expression of stable gp140 protein in its oligomeric conformation. Therefore, the constructs employing the protease cleavage site mutation were used for the derivation of stable CHO cell lines for protein production. Cell lines were also

derived for the monomeric US4 gp120. Expression for these stable CHO cell lines ranged from 1-15 ug/ml of secreted Env glycoprotein.

The antigens in the immunogenic compositions will typically be in the form of HIV proteins. The proteins can, of course, be prepared by various means (*e.g.* native expression, recombinant expression, purification from cell culture culture, chemical synthesis *etc.*) and in various forms (*e.g.* native, fusions *etc.*). They are preferably prepared in substantially pure form (*i.e.* substantially free from other bacterial or host cell proteins).

Other antigens which may advantageously be included in compositions of the invention are:

- a protein antigen from *N.meningitidis* serogroup B, such as those in refs. International patent application WO99/24578; International patent application WO99/36544; International patent application WO99/57280; International patent application WO00/22430; Tettelin *et al.* (2000) *Science* 287:1809-1815; International patent application WO96/29412; Pizza *et al.* (2000) *Science* 287:1816-1820 with protein '287' (see below) and derivatives (*e.g.* 'ΔG287') being particularly preferred.
- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in refs. International patent application PCT/IB01/00166; Bjune *et al.* (1991) *Lancet* 338(8775):1093-1096; Fukasawa *et al.* (1999) *Vaccine* 17:2951-2958; Rosenqvist *et al.* (1998) *Dev. Biol. Stand.* 92:323-333; *etc.*
- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. i from serogroup C [see also ref. Costantino *et al.* (1999) *Vaccine* 17:1251-1263].
- a saccharide antigen from *Streptococcus pneumoniae* [*e.g.*, Watson (2000) *Pediatr Infect Dis J* 19:331-332; Rubin (2000) *Pediatr Clin North Am* 47:269-285, v.; Jedrzejas (2001) *Microbiol Mol Biol Rev* 65:187-207.
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* Bell (2000) *Pediatr Infect Dis J* 19:1187-1188; Iwarson (1995) *APMIS* 103:321-326.
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* Iwarson (1995) *APMIS* 103:321-326; Gerlich *et al.* (1990) *Vaccine* 8 Suppl:S63-68 & 79-80.]

- an antigen from hepatitis C virus [*e.g.* Hsu *et al.* (1999) *Clin Liver Dis* 3:901-915.].

- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. Gustafsson *et al.* (1996) *N. Engl. J. Med.* 334:349-355; Rappuoli *et al.* (1991) *TIBTECH* 9:232-238.]

-a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 3 of *Vaccines* (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0.] *e.g.* the CRM_{197} mutant [*e.g.* Del Guidice *et al.* (1998) *Molecular Aspects of Medicine* 19:1-70.].

- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 4 of *Vaccines* (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0.].

- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* Costantino *et al.* (1999) *Vaccine* 17:1251-1263].

- an antigen from *N.gonorrhoeae* [*e.g.* International patent application WO99/24578; International patent application WO99/36544; International patent application WO99/57280].

- an antigen from *Chlamydia pneumoniae* [*e.g.* International patent application PCT/IB01/01445; Kalman *et al.* (1999) *Nature Genetics* 21:385-389; Read *et al.* (2000) *Nucleic Acids Res* 28:1397-406; Shirai *et al.* (2000) *J. Infect. Dis.* 181(Suppl 3):S524-S527; International patent application WO99/27105; International patent application WO00/27994; International patent application WO00/37494].

- an antigen from *Chlamydia trachomatis* [*e.g.* International patent application WO99/28475].

- an antigen from *Porphyromonas gingivalis* [*e.g.* Ross *et al.* (2001) *Vaccine* 19:4135-4142].

- polio antigen(s) [*e.g.* Sutter *et al.* (2000) *Pediatr Clin North Am* 47:287-308; Zimmerman & Spann (1999) *Am Fam Physician* 59:113-118, 125-126] such as IPV or OPV.

- rabies antigen(s) [*e.g.* Dreesen (1997) *Vaccine* 15 Suppl:S2-6] such as lyophilised inactivated virus [*e.g.* 77, RabAvert™].

- measles, mumps and/or rubella antigens [*e.g.* chapters 9, 10 & 11 of *Vaccines* (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0].

- influenza antigen(s) [*e.g.* chapter 19 of [63] *Vaccines* (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0.], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* McMichael (2000) *Vaccine* 19 Suppl 1:S101-107].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* Schuchat (1999) *Lancet* 353(9146):51-6; International patent application PCT/GB01/04789].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* International patent application PCT/GB01/04789; Dale (1999) *Infect Dis Clin North Am* 13:227-43, viii; Ferretti *et al.* (2001) *PNAS USA* 98: 4658-4663].
- an antigen from *Staphylococcus aureus* [*e.g.* Kuroda *et al.* (2001) *Lancet* 357(9264):1225-1240; see also pages 1218-1219].
- LTK63 and LTR72 (discussed *infra*).

Where a saccharide or carbohydrate antigen is included, it is preferably conjugated to a carrier protein in order to enhance immunogenicity [Ramsay *et al.* (2001) *Lancet* 357(9251):195-196. See also: Lindberg (1999) *Vaccine* 17 Suppl 2:S28-36; *Conjugate Vaccines* (eds. Cruse *et al.*) ISBN 3805549326, particularly vol. 10:48-114 *etc.*]. Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria, cholera, E. coli heat labile or tetanus toxoids. The CRM_{197} diphtheria toxoid is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [European patent application 0372501], synthetic peptides [European patent applications 0378881 & 0427347], heat shock proteins [International patent application WO93/17712], pertussis proteins [International patent application WO98/58668; see also EP-0471177], protein D from *H.influenzae* [International patent application WO00/56360.], toxin A or B from *C.difficile* [International patent application WO00/61761], *etc.* Any suitable conjugation reaction can be used, with any suitable linker where necessary.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus

antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

As used herein, the terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the proteins or errors due to PCR amplification.

The phrase "antigen", as used herein, refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term "antigen" denotes both subunit antigens, (i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein.

Furthermore, for purposes of the present invention, an "antigen" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, specific effector cells, such as B and plasma cells as well as cytotoxic T cells, against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. In addition, a chemokine response may be induced by various white blood or endothelial cells in response to an administered antigen.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-

specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376. Recent methods of measuring cell-mediated immune response include measurement of intracellular cytokines or cytokine secretion by T-cell populations (*e.g.*, by ELISPOT technique), or by measurement of epitope specific T-cells (e.g., by the tetramer technique)(reviewed by McMichael, A.J., and O'Callaghan, C.A., *J. Exp. Med.* 187(9):1367-1371, 1998; Mcheyzer-Williams, M.G., et al, *Immunol. Rev.* 150:5-21, 1996; Lalvani, A., et al, *J. Exp. Med.* 186:859-865, 1997).

Thus, an immunological response as used herein may be one that stimulates the production of CTLs, and/or the production or activation of helper T- cells. The production of chemokines and/or cytokines may also be stimulated. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor, cytotoxic, or helper T-cells and/or T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

The compositions of this invention also include a mucosal adjuvant.

As used herein, the phrase "mucosal adjuvant" refers to an adjuvant suitable for mucosal delivery. Preferably, the adjuvant is suitable for intranasal, intra-vaginal or intra-rectal delivery.

The phrase "mucosal delivery" refers to delivery or administration of a pharmaceutical composition or a vaccine via one or more mucosal routes. Mucosal routes suitable for use in this invention include but are not limited to oral, intranasal, intragastric, pulmonary, intestinal, rectal, ocular, and vaginal. In a preferred embodiment, the mucosal route is intranasal.

Where the mucosal delivery is by an intranasal route, the vaccine of the invention may be in the form of a nasal spray, nasal drops, gel or powder. See Ref. [8].

Where the vaccine is for oral route, for instance, it may be in the form of tablets or capsules (optionally enteric-coated), liquid, transgenic plants, etc. (*see also* Ref. [7] and Chapter 17 of Ref [19]).

Mucosal adjuvants suitable for use in the invention include but are not limited to *E.coli* heat-labile enterotoxins ("LT"), or detoxified mutants thereof, such as the K63 or R72 mutants. *See e.g.* Chapter 5 of ref. [9]. Other mucosal adjuvants suitable for use in the invention include cholera toxin ("CT") or detoxified mutants thereof (See e.g . Chapter 5 of ref. [9]) and microparticles (i.e., a particle of about 100 nm to about 150 μm in diameter, more preferably about 200 nm to about 30μm in diameter, and still more preferably about 500 nm to about 10 μm in diameter) formed from materials that are biodegradable and non-toxic (e.g., a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.).

Preferably, the mucosal adjuvants of the invention are LT mutants such as the R72 and the K63 mutants. See e.g., ref. [15].

Microparticles can also be used in the invention as mucosal adjuvants. These are preferably derived from a poly(α-hydroxy acid), in particular, from a poly(lactide) ("PLA"), a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly(D,L-lactide-co-glycolide) ("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials which have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide

ratios, the selection of which will be largely a matter of choice, depending in part on the coadministered antigen.

The antigen may be entrapped within the microparticles, or may be adsorbed onto their surfact. PLG microparticles are discussed in further detail in ref. [16], in chapter 13 of ref. [17], and in chapters 16 & 18 of ref. [19].

One or more HIV antigens can be used in the vaccine and methods of this invention. For instance, Ogp140 antigens can be used with gag antigens. In this embodiment, the Ogp140-containing and the gag-containing microparticles may be a mixture of two distinct populations of microparticles, the first containing Ogp140 and the second containing gag. Alternatively, the microparticles may be present as a single population, with Ogp140 and gag (and any further antigens) distributed evenly.

LT mutants may advantageously be used in combination with microparticle-entrapped antigen, resulting in significantly enhanced immune responses.

Optionally, an immuno-modulatory factor may be added to the pharmaceutical composition.

As used here, an "immuno-modulatory factor" refers to a molecule, for example a protein that is capable of modulating an immune response. Non-limiting examples of immunomodulatory factors include lymphokines (also known as cytokines), such as IL-6, TGF-β, IL-1, IL-2, IL-3, etc.); and chemokines (*e.g.*, secreted proteins such as macrophage inhibiting factor). Certain cytokines, for example TRANCE, flt-3L, and a secreted form of CD40L are capable of enhancing the immunostimulatory capacity of APCs. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1γ, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO), CD40 ligand (CD40L), tumor necrosis factor-related activation-induced cytokine (TRANCE) and flt3 ligand (flt-3L).

Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). The sequences of many of these molecules are also available, for example, from the GenBank database. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or mutants thereof) and nucleic acid encoding these molecules are intended to be used within the spirit and scope of the invention.

The compositions of the invention will typically be formulated with pharmaceutically acceptable carriers or diluents. As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier for administration of the antigens which does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee et al. (1997) *J Microencapsul.* 14(2):197-210; O'Hagan et al. (1993) *Vaccine* 11(2):149-54. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc., as well as toxins derived from *E. coli.*

Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of acceptable excipients is available in the well-known *Remington's Pharmaceutical Sciences.*

Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

Further, the compositions described herein can include various excipients, adjuvants, carriers, auxiliary substances, modulating agents, and the like. Preferably, the compositions will include an amount of the antigen sufficient to mount an immunological response. An appropriate effective amount can be determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials and will generally be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 300 μg, of particle/antigen.

As set forth above, preferred mucosal adjuvants for use in this invention include detoxified mutants of E. coli heat labile toxin (LT), such as LTR72 and LTK63.

Additional adjuvants may also be used in the invention. Such adjuvants include, but are not limited to: (1) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), beta chemokines (MIP, 1-alpha, 1-beta Rantes, etc.); (2) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International

Publication Nos. W093/13202; W092/19265; WO 95/17211; WO 98/18928 and WO 01/22993); and (3) other substances that act as immunostimulating agents to enhance the effectiveness of the composition; oligodeoxy nucleotides containing immunostimulatory CpG motifs (Cpg); or combinations of any of the above.

2. Methods

The compositions disclosed herein can be administered to a subject to generate an immune response. Preferably, the composition can be used as a vaccine to treat or prevent HIV infection.

As used herein, "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

The compositions will include "immunologically effective amounts" of HIV antigen *i.e.* amounts sufficient to raise a specific immune response or, more preferably, to treat, reduce, or prevent HIV infection. An immune response can be detected by looking for antibodies to the HIV antigen used (*e.g.* IgG or IgA) in patient samples (*e.g.* in blood or serum, in mesenteric lymph nodes, in spleen, in gastric mucosa, and/or in faeces). The precise effective amount for a given patient will depend upon the patient's age, size, health, the nature and extent of the condition, the precise composition selected for administration, the patient's taxonomic group, the capacity of the patient's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating physician's assessment of the medical situation, and other relevant

factors. Thus, it is not useful to specify an exact effective amount in advance, but the amount will fall in a relatively broad range that can be determined through routine trials, and is within the judgement of the clinician. For purposes of the present invention, an effective dose will typically be from about 0.01mg/kg to 50mg/kg in the individual to which it is administered.

3. Techniques and Further Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature *eg.* Sambrook *Molecular Cloning; A Laboratory Manual, Second Edition* (1989); *DNA Cloning, Volumes I and II* (D.N Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed, 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Transcription and Translation* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller & M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer & Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, (1987) *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), and *Handbook of Experimental Immunology, Volumes I-IV* (Weir & Blackwell eds 1986).

The term "comprising" means "including" as well as "consisting", so a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

A composition containing X is "substantially free" from Y when at least 85% by weight of the total X+Y in the composition is X. Preferably, X comprises at least ~90% by weight of the total of X+Y in the composition, more preferably at least ~95% or even 99% by weight.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

EXAMPLE

The following example is offered by way of illustration, and not by way of limitation.

This example demonstrates the induction of an immune response in rhesus macaques through mucosal immunization with HIV-1 gag and HIV-1 Ogp140.

Two groups of rhesus macaques were immunized intranasally (IN) with a combination of HIV-1gag (p24) and HIV-1 Ogp. Each group contained two animals. The animals in Group One were immunized in the presence of LTK63. The animals in Group Two were immunized in the presence of LTR72. The formulations used for each group are set forth below in Table 1.

Table 1: Immunization Formulations

	Ogp140	gag	LTK63	LTR72
Group One	300μg	300μg	100μg	--
Group Two	300μg	300μg	100μg	--
Group Three	300μg	300μg	--	100μg
Group Four	300μg	300μg	--	100μg

An antibody mediated response was observed after the course of five immunizations. Serum IgG titers for each animal two weeks post the fourth immunization (2wp4) and two weeks post the fifth immunization (2wp5) are set forth in Tables 2 and 3 below. Table 2 contains the anti-Ogp140 antibody titers. Table 3 contains the anti-gag (p24) antibody titers. Vaginal wash IgA titers for each animal are set forth in Tables 4 and 5 below. Table 4 contains the anti-Ogp antibody titers. Table 5 contains the anti-gag (p24) antibody titers.

Table 2: Serum Anti-Ogp140 IgG Titers

Group One		2wp4	2wp5
	Animal One	2,165	4,996
	Animal Two	21,573	6,528
Group Two			
	Animal One	464	712
	Animal Two	7,425	3,665

Table 3: Serum Anti-gag (p24) IgG Titers

Group One		2wp4	2wp5
	Animal One	44	1326
	Animal Two	553	813
Group Two			
	Animal One	30	164
	Animal Two	353	4877

Table 4: Vaginal Wash Anti-Ogp140 IgA Titers

Group One		2wp4	2wp5
	Animal One	1333	35
	Animal Two	154	135
Group Two			
	Animal One	95	217
	Animal Two	86	335

Table 5: Vaginal Wash Anti-gag (p24) IgA Titers

Group One		2wp4	2wp5
	Animal One	16	2
	Animal Two	2.5	1.5
Group Two			
	Animal One	17	4
	Animal Two	15.5	67